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Mutations in the Core Promoter Region of Hepatitis B Virus in Patients With Chronic Hepatitis B

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The core promoter region of hepatitis B virus genomes regulates transcription of the precore and pregenomic mRNAs encoding hepatitis B e antigen (HBeAg) and core antigen that contain target epitopes for cytotoxic T lymphocytes. The prevalence and clinical significance of mutations in this region were investigated. DNA was extracted from six asymptomatic carriers positive for HBeAg, eight asymptomatic carriers positive for an anti-HBe antibody, and 24 patients with chronic liver disease. The core promoter and precore regions of hepatitis B virus genomes were amplified by polymerase chain reaction, and predominant sequences were determined by direct sequencing. Mutations were found in none of the HBeAg-positive asymptomatic carriers but in all of the anti-HBe-positive asymptomatic carriers and the patients with chronic liver disease. Especially, A to T mutations at nucleotide 1762 and G to A mutations at nucleotide 1764 were found in five anti-HBe-positive asymptomatic carriers, and 22 patients with chronic liver disease. These two mutation hot spots were located within binding sites of the nuclear factors, and nucleotide 1762 was also involved in the A, T rich sequence that is located 28 base pairs upstream of the precore mRNA initiation site. Serum HBeAg and DNA polymerase levels were significantly lower in patients with these mutations than those without these mutations, and five individuals with these mutations were positive for anti-HBe despite the absence of the precore stop codon mutation. These mutants may be selected by host immune response to HBeAg and/or core antigen.

KEY WORDS: hepatitis B virus, core promoter region, mutation, transcription

INTRODUCTION

Hepatitis B e antigen (HBeAg) and core antigen (HBcAg) contain target epitopes of cytotoxic T cells (CTL) that are suggested to mediate hepatocellular injury [Mondelli et al., 1982; Vento et al., 1985; Ferrari et al., 1987; Milich et al., 1987; Pignatelli et al., 1987; Bertoletti et al., 1991]. A precore stop codon mutation, which prevents complete translation of HBeAg, appears along with seroconversion from HBeAg-positive state to anti-HBe antibody-positive state [Carman et al., 1989; Okamoto et al., 1990; Brunetto et al., 1991], and core gene mutations or deletions that alter amino acid residues of HBcAg are associated with severe liver damage [Wakita et al., 1991; Ehata et al., 1992]. Hepatitis B virus (HBV) with these mutations may diminish antigen production or alter antigen epitopes, and may be selected under host immune pressure.

HBV is a partially double-stranded DNA virus of 3.2 Kb. Upon infection, HBV-DNA undergoes a repair process and forms closed circular DNA in hepatocytes. Transcription of this DNA produces 3.5 Kb precore mRNA and pregenomic mRNA, from which HBeAg, HBcAg and polymerase proteins are translated. The pregenomic RNA is also packaged into core particles and serves as a template for DNA replication [Summers and Mason, 1982; Seeger et al., 1986; Will et al., 1987]. The transcription of the precore and pregenomic mRNAs is regulated by the core promoter region that locates upstream of the initiation site of the precore and pregenomic mRNAs [Honigwachs et al., 1989; Lopez-Cabrera et al., 1990; Yuh et al., 1992]. Since a single nucleotide change in the promoter region could alter significantly its activity [Phares and Herr, 1991; Steffy and Weir, 1991], mutations in the core promoter region may alter the transcrip-

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TABLE I. Clinical Characteristics of Asymptomatic HBV Carriers

Patient no. and age/sex	ALT (IU/l)	DNA-p[a] (cpm)	HBeAg(CI)/ HBeAb[b]	Mutations in CP[c]	Mutations in nt 1762,64	Precore stop codon
HBeAg-positive asymptomatic carriers						
1. 20/M	29	8,841	+(50)/−	−	−	−
2. 23/M	25	7,881	+(55)/−	−	−	−
3. 19/F	20	1,657	+(47)/−	−	−	−
4. 31/F	26	7,951	+(62)/−	−	−	−
5. 41/F	22	10,528	+(52)/−	−	−	−
6. 5/M	20	1,249	+(44)/−	−	−	−
Anti-HBe-positive asymptomatic carriers						
7. 57/F	24	14	−/+	+	+	−
8. 60/M	25	11	−/+	+	−	+
9. 19/F	22	0	−/+	+	+	+
10. 39/M	18	0	−/+	+	+	−
11. 51/M	31	0	−/+	+	+	+
12. 39/M	16	5	−/+	+	−	+
13. 23/M	18	0	−/+	+	−	+
14. 25/F	34	0	−/+	+	+	+

[a]DNA-p, hepatitis B virus DNA polymerase.
[b]HBeAg(CI)/Ab, serum hepatitis B e antigen (cut-off index value of hepatitis B e antigen)/antibody to hepatitis B e antigen.
[c]CP, core promoter region.

tion of mRNAs and thereby the expression of immunodominant HBeAg and HBcAg.

The prevalence and clinical significance of mutations in the core promoter region of HBV genomes were investigated in asymptomatic carriers and in patients with chronic liver disease.

MATERIALS AND METHODS

Materials

Sera were obtained from 38 patients with chronic HBV infection, who were followed routinely at our hospitals. Informed consent was obtained from each patient. Six were HBeAg-positive asymptomatic carriers who had no history of liver disease, and eight were anti-HBe-positive asymptomatic carriers whose liver function was normal persistently for at least for 24 months. Twenty-four were patients with chronic liver disease; 11 were positive for HBeAg, 10 were positive for anti-HBe, and 3 were positive for both HBeAg and anti-HBe. Clinical characteristics of the patients are summarized in Tables I and II. Sera were stored at −70°C until DNA extraction. HBeAg was measured by radioimmunoassay and an anti-HBe antibody was assayed by enzyme immunoassay. HBV-DNA polymerase activity was assayed by the H^3-thymidine incorporation method. Hepatitis C virus antibodies were measured by a second-generation enzyme immunoassay, and were not found in any of the patients.

Primer Synthesis

Nested primers for polymerase chain reaction (PCR) were synthesized by a DNA synthesizer model 391 (Applied Biosystems Japan, Tokyo, Japan) based on the published HBV sequence of pJDW233 [Okamoto et al., 1988]. A nucleotide sequence of T7 promoter primer (24 nucleotides) was added to the 5′ end of the "inner" sense primer and a nucleotide sequence of pUC/M13 reverse primer (24 nucleotides) was added to the 5′ end of the "inner" antisense primer. The T7 promoter primer and the pUC/M13 reverse primer (Promega, Madison, WI) were used as sequencing primers. Sequences of the primers are as follows: "outer" sense primer; 5′ACGTCGCATGGAGACCACCG3′ (nucleotides 1601–1620) "outer" antisense primer; 5′GGAAAGAAGTCAGAAGGCAAA3′ (nucleotides 1954–1974) "inner" sense primer; 5′TAATACGACTCACTATAGGGGAGACCACCGTGAACGCCCA3′ (T7 promoter primer sequence and nucleotides 1611–1630) "inner" anti-sense primer; 5′TCACACAGGAAACAGCTATGACGAGAGTAACTCCACAGAAGCT3′ (PUC/M13 reverse primer sequence and nucleotides 1930–1950) T7 promoter primer; 5′TAATACGACTCACTATAGGG3′ pUC/M13 reverse primer; 5′TCACACAGGAAACAGCTATGAC3′.

Amplification of HBV Gene

DNA was extracted from sera by the sodium hydroxide method [Kaneko et al., 1989]. Briefly, 10 μl of serum were treated with 0.1 N sodium hydroxide in a volume of 20 μl at 37°C for 60 min and then neutralized with hydrochloric acid. A 2 μl aliquot was then amplified by PCR in a 50 μl mixture with 10 pmol of the "outer" primer sets for 35 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The second-round PCR was carried out on 1 μl of the first PCR products in a 50 μl mixture with 10 pmol of each "inner" primer for 35 cycles under the same reaction condition as the first-round PCR. The second PCR products were electrophoresed through agarose gel and visualized under ultraviolet light. Negative controls were included in each PCR round and false positive results were avoided by the strict application of the contamination prevention guidelines [Kwok and Higuchi, 1989].

TABLE II. Clinical Characteristics of Patients with Chronic Liver Disease

Patient no. and age/sex	ALT (IU/l)	DNA-p[a] (cpm)	HBeAg(CI)/ HBeAb[b]	Mutations in		Precore stop codon	Liver histology
				CP[c]	nt 1762,64		
15. 4/F	45	45	+(28)/−	+	+	−	ND[d]
16. 11/F	46	55	+(43)/−	+	+	−	ND
17. 37/M	110	10,468	+(48)/−	+	+	−	CAH[f]
18. 44/F	154	508	+(33)/−	+	+	−	CAH
19. 50/M	170	368	+(40)/−	+	+	−	CAH
20. 61/M	75	34	+(39)/−	+	+	−	LC
21. 30/M	2037	379	+(22)/−	+	+	−	CAH
22. 24/M	910	34	+(22)/−	+	+	+	CAH
23. 43/M	1601	2,828	+(49)/−	+	+	−	ND
24. 45/F	204	309	+(46)/−	+	+	−	CIH[e]
25. 57/M	171	774	+(59)/−	+	+	−	LC
26. 10/M	107	88	+/+	+	+	+	ND
27. 57/M	103	1,024	+/+	+	+	+	CAH
28. 54/F	75	68	+/+	+	+	+	CAH
29. 46/F	387	279	−/+	+	+	−	CAH
30. 59/M	73	6	−/+	+	+	−	LC
31. 42/M	150	73	−/+	+	+	−	LC
32. 35/F	74	167	−/+	+	+	+	CAH
33. 55/M	88	347	−/+	+	+	+	CAH
34. 47/F	101	18	−/+	+	+	+	CAH
35. 37/M	388	10	−/+	+	+	+	CAH
36. 38/M	85	28	−/+	+	+	+	CAH
37. 56/M	578	960	−/+	+	−	+	LC[g]
38. 57/M	330	22	−/+	+	−	+	CAH

[a]DNA-p, hepatitis B virus DNA polymerase.
[b]HBeAg(CI)/Ab, serum hepatitis B e antigen (cut-off index value of hepatitis B e antigen)/antibody to hepatitis B e antigen.
[c]CP, core promoter region.
[d]ND, not determined.
[e]CIH, chronic inactive hepatitis.
[f]CAH, chronic active hepatitis.
[g]LC, liver cirrhosis.

Direct-Sequencing of PCR Products

Residual dNTPs and primers were removed from the PCR products using a centrifugation filtration column (Suprec-02: Takara, Kyoto, Japan) and adjusted to a final volume of 15 μl with Tris-EDTA buffer. Nucleotide sequences of the PCR products were directly determined using Taq DyeDeoxy Terminator Cycle Sequencing Kits in an automated DNA sequencer model 373A (Applied Biosystems). In brief, 7 μl of purified PCR products were mixed with 3.2 pmol of the sequencing primers, 1 μl of each DyeDeoxy Terminator (A, G, C, T), 1 μl of a dNTP mixture (750 μM dITP, 150 μM dATP, 150 μM dTTP, and 150 μM dCTP), 4 units of AmpliTaq DNA polymerase, and 4μl of the reaction buffer (400 mM Tris-HCl, 10 mM $MgCl_2$, and 100 mM $(NH_4)_2SO_4$; pH 9.0) in a total volume of 20 μl. Cycle sequencing reactions were performed for 25 cycles, each cycle consisting of rapid thermal ramp to 96°C, 96°C for 30 sec, rapid thermal ramp to 50°C, 50°C for 25 sec, rapid thermal ramp to 60°C, and 60°C for 4 min. An excess of DyeDeoxy Terminators was removed from the completed reaction mixture using a Centri-Sep spin column (Princeton Separations, Adelphia, NJ). Samples were dried in a vacuum centrifuge, dissolved in a loading buffer (5 μl deionized formamide and 1 μl of 50 mM EDTA, pH 8.0), and loaded onto an Applied Biosystems 373A DNA sequencer according to the instructions.

Comparison of the Nucleotide Sequences With Previously Reported HBV Sequences

In order to determine the reference sequence of the core promoter region of HBV genomes, multiple alignments of the nucleotide sequences were made and amino acid residues were deduced from full-length HBV clones of genotypes A, B, C, and D described previously [Okamoto et al., 1986a,b, 1988; Pasek et al., 1979; Galibert et al., 1979; Valenzuela et al., 1980; Fujiyama et al., 1983; Ono et al., 1983; Kobayashi and Koike, 1984; Bichko et al., 1985; Sastrosoewignjo et al., 1987; Vaudin et al., 1988; Rho et al., 1989; Takemura et al., 1990; Lai et al., 1991; Gan et al., 1987; Qi et al., 1989] using a computer software DNASIS-Mac version 2.0 (Hitachi Software Engineering Co., Japan). Two clones implicated in fulminant hepatitis [Ogata et al., 1993; Hasegawa et al., 1994] and three clones derived from anti-HBe-positive patients with chronic liver disease [Loncarevic et al., 1990; Tong et al., 1990; Blum et al., 1991] were not included in the alignment. Sequences that were observed in more than 70% of the clones at each nucleotide and amino acid position were defined as consensus sequences, and sequences different from the consensus sequences were regarded as mutations. When such consensus sequences could not be determined, sequences that were observed in less than 20% of the reported clones were regarded as mutations.

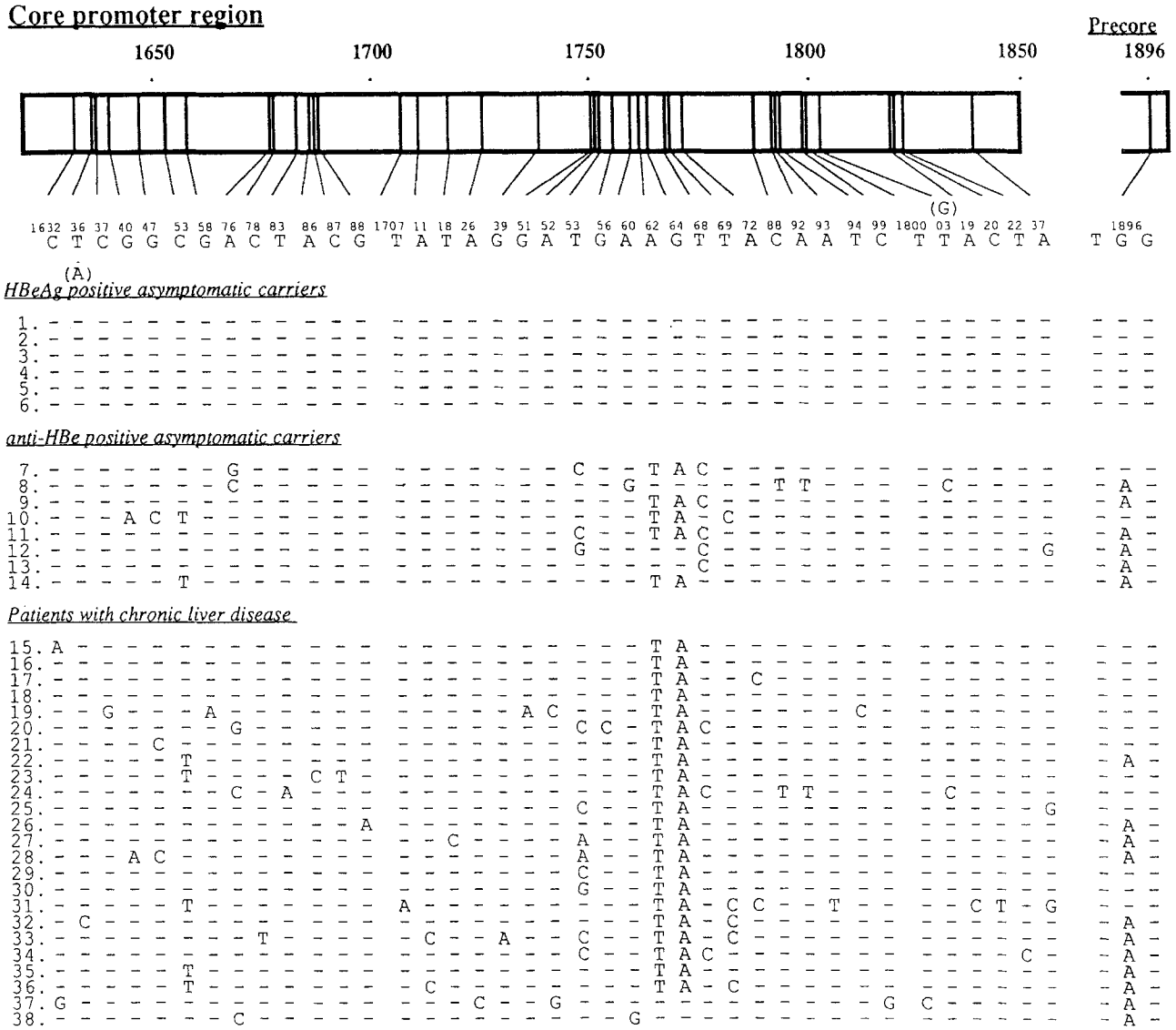

Fig. 1. Nucleotide sequences in the core promoter region. Positions of nucleotide mutations are indicated by vertical lines along the core promoter region of HBV genome. The consensus nucleotide sequence of genotype C is shown in the first line. The consensus nucleotide sequence of genotypes A and B, that are different from genotype C, is indicated in parentheses in the upper and lower line, respectively. Dashes represent nucleotide sequences identical to these reference sequences. In patients with chronic liver disease, and anti-HBe-positive asymptomatic carriers, 131 rare nucleotide mutations are found at 39 genome sites, while none is found in HBeAg-positive asymptomatic carriers. Especially, nucleotides 1762 and 1764 are hot spots of mutations.

Statistical Analysis

The data were analyzed using Mann-Whitney U test, and the P value less than 0.05 was regarded as significant.

RESULTS

Nucleotide Sequences of Core Promoter Gene

Sequencing of the core promoter and precore regions revealed that carrier 13 was infected with genotype A; carriers 12 and 17 were infected with genotype B, and the remainders were infected with genotype C. The nucleotide sequences are aligned in Figure 1. No mutations in the core promoter region were detected in HBeAg-positive asymptomatic carriers, while mutations were invariably found in patients with chronic liver disease and anti-HBe-positive asymptomatic carriers. Nucleotides 1762 and 1764 were hot spots of mutations; an A to T mutation at nucleotide 1762 and a G to A mutation at nucleotide 1764 occurred in five out of eight anti-HBe-positive asymptomatic carriers (63%), and 22 out of 24 patients with chronic liver disease (92%); 11/11 HBeAg-positive patients showed the mutations (100%) which also occurred in 3 out of 3 patients positive for HBeAg and anti-HBe (100%) and 8 out of 10 cases positive for anti-HBe. Nucleotides 1762 and 1764 were located within the binding site of the rat liver nuclear

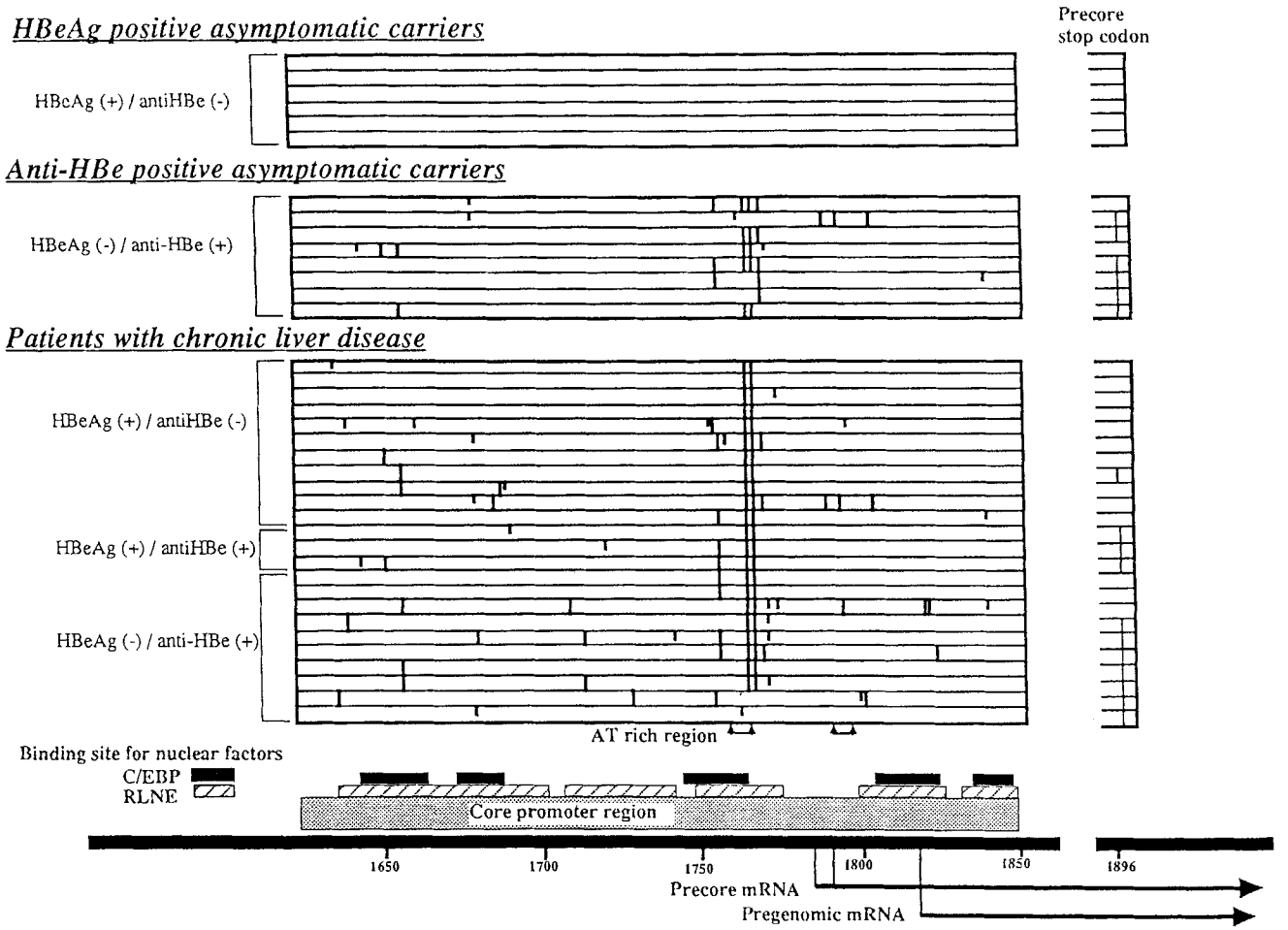

Fig. 2. Schematic demonstration of mutations in the core promoter region. The locations of mutations and binding sites of the nuclear factors are schematically summarized. Positions of nonsilent mutations are indicated by long vertical lines and silent mutations are indicated by short vertical lines along the core promoter region of HBV genome. Mutations are invariably found in patients with chronic hepatitis and anti-HBe-positive asymptomatic carriers, whereas none was found in HBeAg-positive asymptomatic carriers. Hot spot mutations at nucleotides 1762 and 1764 are located within the reported binding site of the rat liver nuclear extract (RLNE) or CCAAT/enhancer binding proteins (C/EBP; [Lopez-Cabrera et al., 1990]). Nucleotide 1762 involves the A, T rich sequence (TTAAA:nucleotides 1758–1762) 28 base pairs upstream of the precore mRNA initiation site (nucleotides 1783–1784 and 1789–1791). There is no mutation directly affecting the initiation sites for precore RNA (nucleotides 1783–1784 and 1789–1791) and pregenomic RNA (nucleotides 1816–1818).

extract (RLNE) and nucleotide 1762 was located within the binding site of the CCAAT/enhancer binding protein (C/EBP) [Lopez-Cabrera et al., 1990]. Moreover, nucleotide 1762 was involved in an A, T rich sequence (TTAAA; nucleotides 1758–1762) that is located 28 base pairs upstream of the initiation site for the precore mRNA (nucleotides 1783–1784 and 1789–1791). Locations of binding sites of the nuclear factors, A, T rich regions and mutations are summarized schematically in Figure 2. There was no mutation affecting directly the initiation sites for the precore mRNA (nucleotides 1783–1784 and 1789–1791) and the pregenomic mRNA (nucleotides 1816–1818). There was no mutation within the 11 base-pair direct repeat DR1 region. A precore stop codon mutation at nucleotide 1896, which converts tryptophan (TGG) to a stop codon (TAG), was not found in HBeAg-positive asymptomatic carriers, but was found in one patient positive for HBeAg (9%), three patients positive for both HBeAg and anti-HBe (100%), and seven patients positive for anti-HBe (70%), and six anti-HBe-positive asymptomatic carriers (75%).

Serum levels of HBeAg and HBV-DNA polymerase were compared between HBeAg-positive individuals with mutations at nucleotides 1762 and 1764 (carriers 15 to 21, 23 to 25), and those without mutations (carriers 1 to 6). Those with a precore stop codon mutation were not included (carriers 22, 26–28). The cut-off index of serum HBeAg was significantly lower in individuals with mutations than those without mutations ($P < 0.05$). The value of HBV-DNA polymerase was also significantly low in individuals with mutations compared with those without mutations ($P < 0.05$; Fig. 3).

Amino Acid Residues of X Protein

Nucleotide sequences that code the 3′ half of X protein were translated into amino acid residues. Deduced

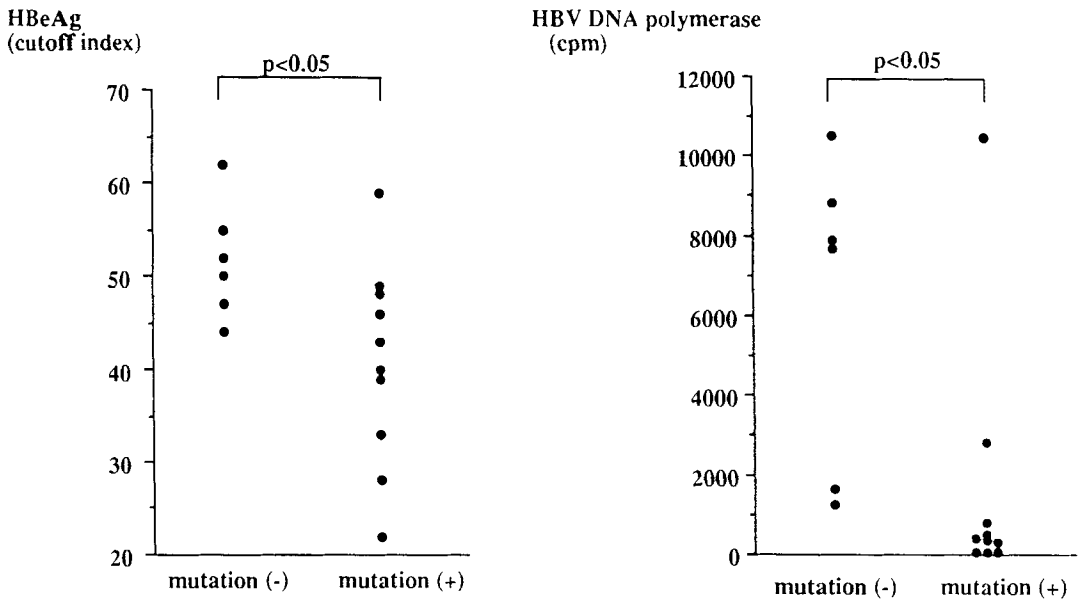

Fig. 3. Comparison of HBeAg and HBV DNA polymerase between HBV carriers with and without mutations in nucleotides 1762 and 1764. Serum levels of HBeAg and HBV-DNA polymerase were compared between HBeAg-positive individuals with (carriers 15 to 21, 23 to 25) and without (carriers 1 to 6) mutations at nucleotides 1762 and 1764. The cut-off index of serum HBeAg was significantly lower in patients with mutations than those without mutations ($P < 0.05$). The value of HBV-DNA polymerase was also significantly low in patients with mutations compared with those without mutations ($P < 0.05$).

amino acid residues are aligned in Figure 4. A mutation from A to T at nucleotide 1762 lead to an alteration of lysine in codon 130 to methionine, and a mutation from G to A at nucleotide 1764 lead to an alteration of valine at codon 131 to isoleucine. These amino acid alterations were located outside the region that is reported to be essential for the transactivating function of X protein [Arii et al., 1992].

DISCUSSION

Two hot spots were identified for nucleotide mutations in the core promoter region of HBV genomes. HBV with these mutations were highly prevalent among patients with chronic liver disease and anti-HBe-positive asymptomatic carriers, while they were not detected in HBeAg-positive asymptomatic carriers.

HBV with a precore stop codon mutation at nucleotide position 1896 is associated with seroconversion to anti-HBe-positive state [Carman et al., 1989; Okamoto et al., 1990; Brunetto et al., 1991] or, in some cases, with fulminant hepatitis [Carman et al., 1991; Kosaka et al., 1991; Hasegawa et al., 1991; Liang et al., 1991; Omata et al., 1991]. Missense mutations [Ehata et al., 1992] or deletions [Wakita et al., 1991] in restricted regions of the core gene are associated with severe hepatocellular injury. These mutations in the precore and core regions of HBV genomes diminish or alter the translation of HBeAg and HBcAg that contain target epitopes of CTL [Mondelli et al., 1982; Vento et al., 1985; Ferrari et al., 1987; Milich et al., 1987; Pignatelli et al., 1987; Bertoletti et al., 1991], and these mutants are selected under host immune pressure. Carman et al. [1995] demonstrated recently clusters of missense mutations in the $CD4^+$ T-helper-cell and B-cell epitopes of the core gene along with seroconversion to anti-HBe, and found a correlation between the number of mutations and active liver disease. Since transcription of the precore and pregenomic mRNAs, which encode HBeAg and HBcAg, respectively, is regulated by the core promoter region, we postulated that mutations in the core promoter region, which may alter the expression of HBeAg and HBcAg at the transcriptional level, may also appear under host immune pressure.

In the present study, mutations were not found in the core promoter region of HBV genomes in the HBeAg-positive asymptomatic carriers. In contrast, various mutations were invariably found in patients with chronic liver disease and anti-HBe-positive asymptomatic carriers. Nucleotides 1762 and 1764 in particular were hot spots for mutations, and an A to T substitution at nucleotide 1762 and a G to A substitution at nucleotide 1764 were found in as many as 92% of HBV genomes from patients with chronic liver disease, and 63% of anti-HBe-positive asymptomatic carriers. Thus, these mutations are highly prevalent among patients with liver disease, or among those who seroconverted to anti-HBe-positive state, but the presence of mutations or HBeAg status may not, per se, be the determining factors of active disease.

These hot spots for mutations were located within the reported binding sites of the nuclear factor RLEN (nucleotides 1747–1775) or C/EBP (nucleotides 1745–1762) [Lopez-Cabrera et al., 1990]. It is well-known that only a single nucleotide change in the promoter region

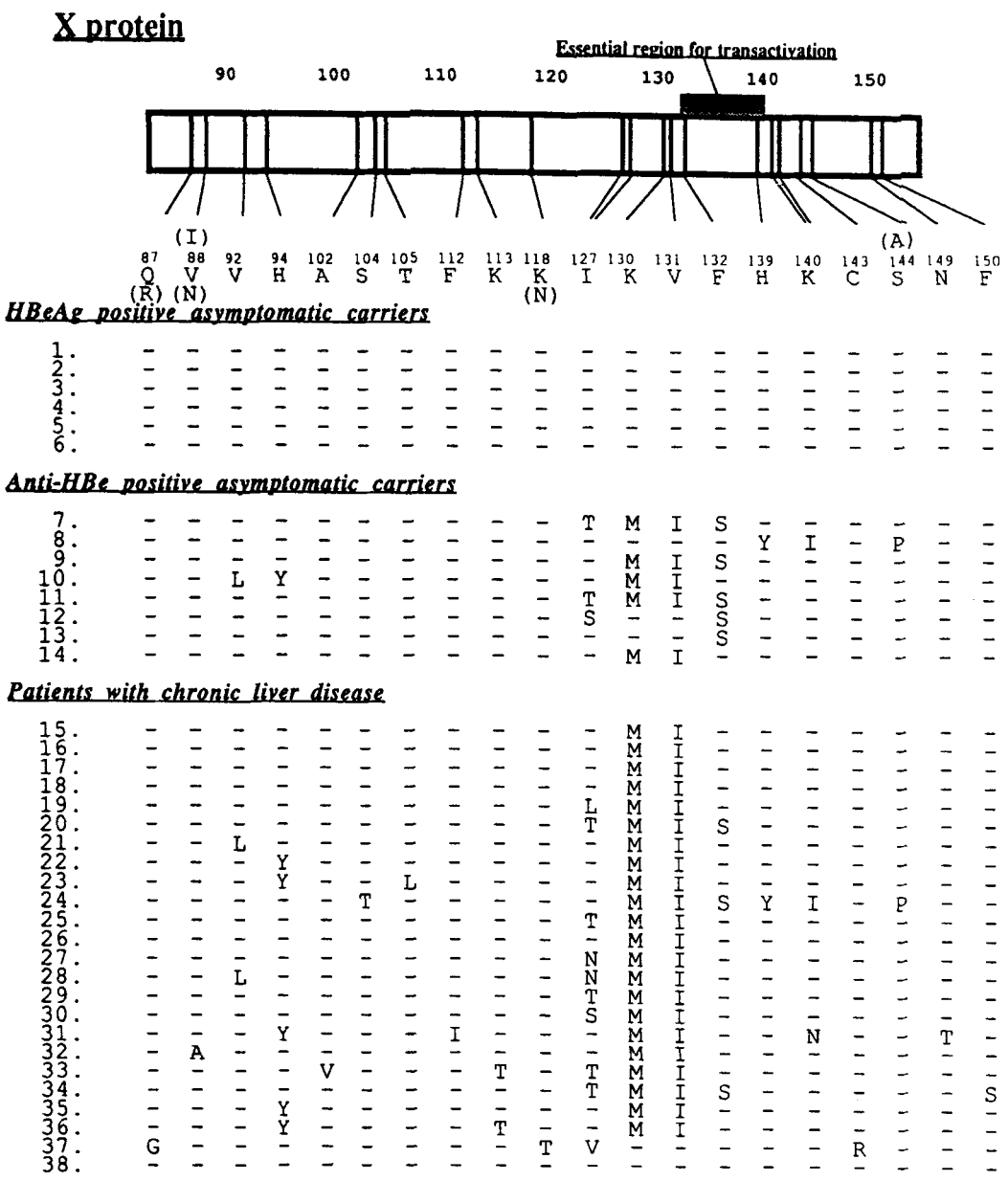

Fig. 4. Amino acid residues in the 3′ half of the X protein. Positions of alterations in deduced amino acid residues are indicated by vertical lines along X protein of HBV. The consensus residue of genotype C is shown in the first line. The consensus residue of genotype A and B, that are different from genotype C, is indicated in parentheses in the upper and lower line, respectively. Dashes represent residues identical to these reference residues. Hot spot mutations at nucleotides 1762 and 1764 altered lysine in codon 130 to methionine, and valine at codon 131 to isoleucine, but they locate outside the region that is reported to be essential for the transactivating function of X protein [Arii et al., 1992].

could reduce its activity through changes in the affinity to the nuclear factors [Phares and Herr, 1991; Steffy and Weir, 1991]. Moreover, an A to T mutation at nucleotide 1762 was involved in the A, T rich sequence that was located 28 base pairs upstream of the precore mRNA initiation site. Point mutations in the TATA sequence motif are reported to decrease specific in vitro transcription drastically by inhibiting RNA polymerase [Wasylyk et al., 1980]. Therefore, these hot spot mutations may reduce transcription of the precore and/or pregenomic mRNAs, followed by a decrease in HBeAg and HBcAg production. In fact, among HBeAg-positive individuals, serum HBeAg levels and HBV-DNA polymerase values were significantly low in patients with mutations at nucleotides 1762 and 1764 compared with carriers without mutations, suggesting the inhibition of precore and pregenomic mRNAs. Among 21 individuals positive for anti-HBe (eight asymptomatic carriers and 13 patients with chronic liver disease), five had a precore stop codon mutation at nucleotide 1896 alone, five had mutations at nucleotides 1762 and 1764 alone, and 11 had both mutations. The observation that five individuals with a muta-

tion at nucleotide 1762 (carriers 7, 9 and 29 to 31) had HBeAg-negative phenotype, in the absence of the precore stop codon mutation, also indicates the significant effect of this mutation on precore mRNA transcription. These findings are comparable with the report that in vitro expression of HBeAg was reduced when transfected with HBV mutants with an 8 base pair deletion in the core promoter region [Preisler-Adams et al., 1994]. Although these results suggest that the presence of such mutations downregulates the transcription, further in vitro trascription studies using a reporter gene under a mutated promoter and the implicated transcription factor, or in vitro transfection studies with mutant HBV using a suitable cell line, are necessary to confirm the effect of mutations in the core promoter region on the level of the expression of viral proteins.

A review of 28 full-length HBV clones reported previously, an A to T substitution at nucleotide 1762 and a G to A substitution at nucleotide 1764 were found in seven clones [Ono et al., 1983; Rho et al., 1989; Ogata et al., 1993; Hasegawa et al., 1994; Loncarevic et al., 1990; Tong et al., 1990; Blum et al., 1991]. Clinical information was available for five clones, and all were isolated from patients with liver disease, one from anti-HBe-positive chronic hepatitis [Tong et al., 1990], two from hepatocellular carcinoma [Loncarevic et al., 1990; Blum et al., 1991], and two from fulminant hepatitis [Ogata et al., 1993; Hasegawa et al., 1994]. Recently, Laskus et al. [1994] reported naturally occurring mutants with deletions or insertions in the core promoter region. More recently, Okamoto et al. [1994] reported the same kind of mutations and suggested that these mutants may induce a HBeAg-negative phenotype. On the other hand, Sato et al. [1995] reported a high prevalence of these mutations in fulminant hepatitis. These findings support the finding that these mutations are found specifically among patients with liver disease, and in some cases related causatively with the development of fulminant hepatitis.

Hot spot mutations at nucleotides 1762 and 1764 altered codons 130 and 131 of X protein. Since the transactivation domains are not yet clearly defined, and the role of X protein as transactivator in vivo is not definitely demonstrated, the influence of these mutations on the transactivation activity of the X protein remains to be elucidated.

In conclusion, HBV with mutations in the core promoter region was prevalent among patients with chronic hepatitis B and anti-HBe-positive asymptomatic carriers. HBV with these mutations, which may reduce the expression of immunodominant viral proteins, may be selected by the host immune response against HBeAg or HBcAg. Further in vivo and in vitro studies are warranted to confirm these conclusions.

REFERENCES

Arii M, Takada S, Koike K (1992): Identification of three essential regions of hepatitis B virus X protein for trans-activation function. Oncogene 7:397–403.

Bertoletti A, Ferrari C, Fiaccadori F, Penna A, Margolskee R, Schlicht HJ, Fowler P, Guilhot S, Chisari FV (1991): HLA class I-restricted human cytotoxic T cells recognize endogenously synthesized hepatitis B virus nucleocapsid antigen. Proceedings of the National Academy of Sciences of the United States of America 88:10445–10449.

Bichko V, Pushko P, Dreilina D, Pumpen P, Gren E (1985): Subtype ayw variant of hepatitis B virus: DNA primary structure analysis. FEBS Letters 185:208–212.

Blum HE, Galun E, Liang TJ, Hasegawa K, Wands JR (1991): Structure and biological characteristics of a naturally occurring precore/core mutant of hepatitis B virus. In Hollinger FB, Lemon SM, Margolis H (eds): "Viral Hepatitis and Liver Disease." Baltimore: Williams & Wilkins, pp 861–865.

Brunetto MR, Giarin MM, Oliveri F, Chiaberge E, Baldi M, Alfarano A, Serra A, Saracco G, Verme G, Will H, Bonino F (1991): Wild-type and e antigen-minus hepatitis B viruses and course of chronic hepatitis. Proceedings of the National Academy of Sciences of the United States of America 88:4186–4190.

Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC (1989): Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet 2:588–591.

Carman WF, Fagan EA, Hadziyannis S, Karayiannis P, Tassopoulos NC, Williams R, Thomas HC (1991): Association of precore genomic variant of hepatitis B virus with fulminant hepatitis. Hepatology 14:219–222.

Carman WF, Thursz M, Hadziyannis S, McIntyre G, Colman K, Gioustoz A, Fattovich G, Alberti A, Thomas HC (1995): Hepatitis B e antigen negative chronic active hepatitis: Hepatitis B virus core mutations occur predominantly in known antigenic determinants. Journal of Viral Hepatitis 2:77–84.

Ehata T, Omata M, Yokosuka O, Hosoda K, Ohto M (1992): Variations in codons 84–101 in the core nucleotide sequence correlate with hepatocellular injury in chronic hepatitis B virus infection. Journal of Clinical Investigation 89:332–338.

Ferrari C, Penna T, Giuberci T, Tong MJ, Ribera E, Ficcadori F, Chisari FV (1987): Intrahepatic, nucleocapsid antigen-specific T cell in chronic active hepatitis B. Journal of Immunology 139:2050–2058.

Fujiyama A, Miyanohara A, Nozaki C, Yoneyama T, Ohtomo N, Matsubara K (1983): Cloning and structural analyses of hepatitis B virus DNAs, subtype adr. Nucleic Acids Research 11:4601–4610.

Galibert F, Mandart E, Fitoussi F, Tiollais P, Charnay P (1979): Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli. Nature (London) 281:646–650.

Gan RB, Chu MJ, Shen LP, Qian SW, Li ZP (1987): The complete nucleotide sequence of the cloned DNA of hepatitis B virus subtype adr in pADR-1. Science in China (Series B) 30:507–521.

Hasegawa K, Huang JK, Wands JR, Obata H, Liang TJ (1991): Association of hepatitis B viral precore mutations with fulminant hepatitis B in Japan. Virology 185:460–463.

Hasegawa K, Huang J, Rogers SA, Blum HE, Liang TJ (1994): Enhanced replication of a hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. Journal of Virology 68:1651–1659.

Honigwachs J, Faktor O, Dikstein R, Shaul Y, Laub O (1989): Liver-specific expression of hepatitis B virus is determined by the combined action of the core gene promoter and enhancer. Journal of Virology 63:919–924.

Kaneko S, Feinstone SM, Miller RH (1989): Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. Journal of Clinical Microbiology 27:1930–1933.

Kobayashi M, Koike K (1984): Complete nucleotide sequence of hepatitis B virus DNA of subtype adr and its conserved gene organization. Gene 30:227–232.

Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshiba M, Tanaka S, Akahane Y, Okamoto H, Tsuda F, Miyakawa Y, Mayumi M (1991): Fulminant hepatitis B: Induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e antigen. Gastroenterology 100:1087–1094.

Kwok S, Higuchi R (1989): Avoiding false positives with PCR. Nature (London) 339:237–238.

Lai ME, Melis A, Mazzoleni AP, Farci P, Balestrieri A (1991): Sequence analysis of hepatitis B virus genome of a new mutant of ayw subtype isolated in Sardinia. Nucleic Acids Research 19:5078.

Laskus T, Rakela J, Tong MJ, Nowicki MJ, Mosley JW, Persing DH (1994): Naturally occurring hepatitis B virus mutants with deletions in the core promoter region. Journal of Hepatology 20:837–841.

Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben PE (1991): A hepatitis

B virus mutant associated with an epidemic of fulminant hepatitis. The New England Journal of Medicine 324:1705–1709.

Loncarevic IF, Zentgraf H, Schroder CH (1990): Sequence of a replication competent hepatitis B virus genome with a preX open reading frame. Nucleic Acids Research 18:4940.

Lopez-Cabrera M, Letovsky J, Hu K-Q, Siddiqui A (1990): Multiple liver-specific factors bind to the hepatitis B virus core/pregenomic promoter: Trans-activation and repression by CCAAT/enhancer binding protein. Proceedings of the National Academy of Sciences of the United States of America 87:5069–5073.

Milich DR, McLachlan A, Moriarty A, Thornton GB (1987): Immune response to hepatitis B virus core antigen (HBcAg): Localization of T cell recognition sites with HBcAg/HBeAg. Journal of Immunology 139:1223–1231.

Mondelli M, Vergani GM, Alberti A, Vergani D, Portmann B, Eddleston ALWF, Williams R (1982): Specificity of lymphocytes cytotoxicity to autologous hepatocytes in chronic hepatitis B virus infection: Evidence that T cells are directed against HBV core antigen expressed on hepatocytes. Journal of Immunology 129:2773–2778.

Ogata N, Miller RH, Ishak KG, Purcell RH (1993): The complete nucleotide sequence of a pre-core mutant of hepatitis B virus implicated in fulminant hepatitis and its biological characterization in chimpanzees. Virology 194:263–276.

Okamoto H, Imai M, Kametani M, Nakamura T, Mayumi M (1986a): Genomic heterogeneity of hepatitis B virus in a 54-year-old woman who contracted the infection through materno-fetal transmission. Japanese Journal of Experimental Medicine 57:231–236.

Okamoto H, Imai M, Shimozaki M, Hoshi Y, Iizuka H, Gotanda T, Tsuda F, Miyakawa Y, Mayumi M (1986b): Nucleotide sequence of a cloned hepatitis B virus genome, subtype ayr: Comparison with genomes of the other three subtypes. Journal of General Virology 67:2305–2314.

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M (1988): Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. Journal of General Virology 65:2575–2583.

Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M (1990): Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. Journal of Virology 64:1298–1303.

Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshiba M, Moriyama K, Tanaka T, Miyazawa Y, Mayumi M (1994): Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. Journal of Virology 68:8102–8110.

Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M (1991): Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. The New England Journal of Medicine 324:1699–1704.

Ono Y, Onda H, Sasada R, Igarashi K, Sugino Y, Nishioka K (1983): The complete nucleotide sequences of the cloned hepatitis B virus DNA; subtype adr and adw. Nucleic Acids Research 11:1747–1757.

Pasek M, Goto T, Gilbert W, Zink B, Schaller H, Nackay P, Leadbetter G, Murray K (1979): Hepatitis B virus genes and their expression in E. coli. Nature (London) 282:575–579.

Phares W, Herr W (1991): Functional similarities between human immunodeficiency virus type 1 and simian virus 40 kB proto-enhancers. Journal of Virology 65:2200–2210.

Pignatelli M, Waters J, Lever A, Iwarson S, Gerety R, Thomas HC (1987): Cytotoxic T-cell response to the nucleocapsid proteins of HBV in chronic hepatitis. Journal of Hepatology 4:15–21.

Preisler-Adams S, Schlayer HJ, Peters T, Hiller B, Gerok W, Rasenack J (1994): In vitro HBV gene expression of immunologically negative HBV variants. Gastroenterology 106:A965.

Qi ZH, Yan J, Xiong WJ, Yuan JG, Song S, Xue CQ, Cai LW (1989): Determination of the complete nucleotide sequence of HBV adr NC-1 DNA. Science in China (Series B) 32:1082–1086.

Rho HM, Kim K, Hyun SW, Kim HS (1989): The nucleotide sequence and reading frames of a mutant hepatitis B virus subtype adr. Nucleic Acids Research 17:2124.

Sastrosoewignjo RI, Omi S, Okamoto H, Mayumi M, Rustam M, Sujudi (1987): The complete nucleotide sequence of HBV DNA clone of subtype adw (pMND 122) from Menado in Sulawesl Island, Indonesia. ICMR Annals 7:51–60.

Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yonomura K, Tsuda F, Tanaka T, Okamoto H, Miyazawa Y, Mayumi M (1995): Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Annals of Internal Medicine 122:241–248.

Seeger C, Ganem D, Vrmus HE (1986): Biochemical and genetic evidence for the hepatitis B virus replication strategy. Science 232:477–484.

Steffy KR, Weir JP (1991): Mutational analysis of two herpes simplex virus type 1 later promotes. Journal of Virology 65:6454–6460.

Summers J, Mason WS (1982): Replication of the genome of a hepatitis B-like virus by revese transcription of an RNA intermediate. Cell 29:403–415.

Takemura F, Ishii T, Fujii N, Uchida T (1990): Complete nucleotide sequence of hepatitis B virus. Nucleic Acids Research 18:4587.

Tong SP, Li JS, Vitvitski L, Trepo C (1990): Active hepatitis B virus replication in the presence of anti-HBe is associated with viral variants containing an inactive pre-C region. Virology 176:596–603.

Valenzuela P, Quiroga M, Zaldivar J, Gray P, Rutter WJ (1980): The nucleotide sequence of the hepatitis B viral genome and the identification of the two major viral genes. In Fields BN, Jaenisch R, Fox CF (eds): "Animal Virus Genetics, ICN-UCLA Symposia on Molecular and Cellular Biology, Volume XVIII." New York: Academic Press, pp 57–70.

Vaudin M, Wolstenholme AJ, Tsiquaye KN, Zuckerman AJ, Harrison TJ (1988): The complete nucleotide sequence of the genome of a hepatitis B virus isolated from a naturally infected chimpanzee. Journal of General Virology 69:1383–1389.

Vento S, Hegarty JE, Alberti A, O'Brien CJ, Alexander GJM, Eddleston ALWF, Williams R (1985): T lymphocytes sensitization to HBcAg and T cell-mediated unresponsiveness to HBsAg in hepatitis B virus-related chronic liver disease. Hepatology 5:192–197.

Wakita T, Kakumu S, Shibata M, Yoshioka K, Ito Y, Shinagawa T, Ishikawa T, Takayanagi M, Morishima T (1991): Detection of pre-C and core region mutants of hepatitis B virus in chronic hepatitis B virus carriers. Journal of Clinical Investigation 88:1793–801.

Wasylyk B, Derbyshire R, Guy A, Molko D, Roget A, Teoule R, Chambon P (1980): Specific in vitro transcription of coalbumin gene is drastically decreased by single-point mutation in T-A-T-A box homology sequence. Proceedings of the National Academy of Sciences of the United States of America 77:7024–7028.

Will H, Reiser T, Weimer T, Pfaff EM, Buscher M, Sprengel R, Cattaneo R, Schaller H (1987): Replication strategy of human hepatitis B virus. Journal of Virology 61:904–911.

Yuh CH, Chang YL, Ting LP (1992): Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. Journal of Virology 66:4073–4084.